THE PREPARATION OF DESLYSYLALANYL PORCINE AND BOVINE INSULINS

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1. Introduction

The treatment of diabetes mellitus can be complicated by the immunogenicity of the insulin preparations used. Local reactions occur in a significant proportion of patients and it has been reported that every subject receiving porcine or bovine insulins shows insulin binding antibodies shortly after the beginning of treatment [1]. These circulating antibodies cause an increase in the insulin requirement over a period of time. Opinions vary as to whether the immunogenicity is due to the actual insulin molecules or because of other components present in the preparations but it is possible that antibody production is an inevitable consequence of repeated injections of a molecular species different from human insulin. Antibodies are presumably directed towards the determinants to which the species is capable of responding. Porcine and human insulins differ by only one amino-acid residue but bovine and human insulins have three residues changed and it is interesting that there is evidence that bovine insulin produces significantly more antibodies than porcine in the diabetic patient [2].

Because of indications that high molecular weight components of insulin preparations were particularly immunogenic, Schlichtkrull et al. [3] and Root et al. [4] sought to remove impurities by ion-exchange chromatography and have shown that the purified insulins were of reduced immunogenicity. The major identified impurities in the insulin preparations appeared to be arginine-31 insulin, desamido insulin and ethyl ester together with proinsulin and intermediates.

The difference between porcine and human insulins is that the C-terminal residue of the B chain is alanine in the porcine and threonine in the human peptide. Carboxypeptidase A will remove the B chain C-terminal amino-acid faster than the A-chain asparagine and it is possible to obtain a des-alanyl peptide with the same sequence as the corresponding human peptide [5]. The use of this insulin in patients resistant to other insulins has been described and significant improvement in the control of the diabetes has been reported in some cases [6]. This supports the view that a readily modified insulin could have a place in diabetic therapy and one such method is described in this letter.

It has been shown that the fruiting body of the fungus *Armillaria Mellea* produces a proteolytic enzyme [7] and that this protease has pronounced specificity towards amide bonds formed on the amino side of a lysine residue (P. L. Walton, personal communication). The specificity has been confirmed and utilised in structural studies on aspartate amino-transferase [8]. Porcine, bovine and indeed most other insulins have a single lysine residue at the penultimate position of the B chain and cleavage at this point would remove a dipeptide containing the C-terminal residue foreign to the human molecule. Additionally the proinsulins have a second lysine residue giving the sequence -lysine-arginine-A-chain [9,10]. An examination of the effect of the *Armillaria Mellea* protease upon insulin preparations was therefore undertaken to investigate whether modified insulins could be obtained which would retain the essential biological activity of insulin [11].

2. Materials

Porcine insulin (NT.51) was obtained from The Boots Company, Nottingham, and bovine insulin (Lot 41895) from Burroughs Wellcome & Co, London. The purified A. Mellea protease was kindly provided by Dr P. L. Walton, ICI Pharmaceuticals Division.

3. Results and discussion

The first experiments were carried out on ten times recrystallised porcine insulin with an enzyme substrate ratio of 1:200 in 0.1 M ammonium bicarbonate at 37°C. Progress of the reaction was followed by dansylation [12], whereby N-terminal amino-acids were identified as their dansyl derivatives, and this showed that the only new N-terminal acid was in fact lysine. A portion of the digest was resolved into two components by paper electrophoresis. The spot showing greatest movement to the cathode at pH 2.1 was found to be the dipeptide lysyl alanine and the second component with only N-terminal glycine and phenylalanine had the amino-acid ratios of the 1–49 unit. When insulin alone was subjected to a standard single column amino-acid analysis programme (Locarte Company, London) it appeared as a single peak about 50 min before the ammonia peak. The two cleavage products appeared as separate peaks in the same region and subsequently an abbreviated programme was used to separate the three components (fig.1). This allowed for rapid estimation of the three components and it was found that even with heavy loading only these three could be seen. Several aspects of this enzymic modification of insulin were followed using the automated analysis programme.

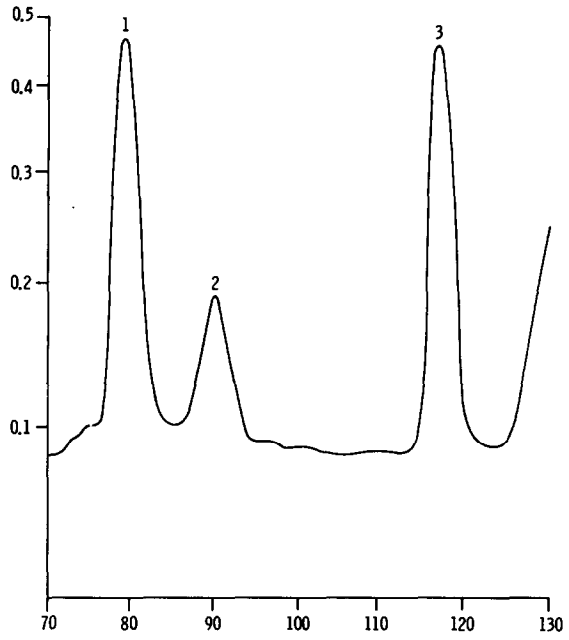

Fig.1. Reproduction of the 570 nm trace showing the separation of insulin (2) from deslysylalanyl insulin (1) and lysyl alanine (3) at 58°C on the 23 cm column of a Locarte amino acid analyser equilibrated with pH 3.25 buffer and then eluted with pH 6.65 buffer.

i) Experiments at different enzyme substrate ratios were carried out at 37°C in 0.1 M ammonium bicarbonate with the insulin concentration at 2 mg/ml. The stock enzyme solution was approximately 0.5 mg/ml but the ratio of enzyme to insulin was established by amino acid analyses of acid hydrolysates from aliquots of the starting solutions. The results showed that cleavage of insulin could be achieved in a few hours with enzyme substrate ratios of less than 1:1000. This ratio was used thereafter.

ii) The influence of pH upon reaction rate was followed using ammonia and acetic acid to give solutions of the desired pH values and, to ensure solubility, the insulin concentration was 400 μg/ml. Aliquots were taken for each pH value from a stock enzyme-insulin solution prepared at 0°. Samples were analysed at 1 and 4 hr and this showed that digestion took place quite satisfactorily from pH 4–9 at 37°C (fig.2).

iii) The reaction was followed at different temperatures, at pH 8.2 and with an insulin concentration of 400 μg/ml. Below ambient temperature digestion was very slow but the rate increased up to 50°C. Above this temperature it was more erratic but with a tendency towards a slower reaction in some experiments.

iv) The effects of different metal salts on the rate of digestion were followed at pH 8.2, 37°C, insulin at 1 mg/ml and with the enzyme substrate ratio held at 1:1000. Profound effects were observed at very high metal concentrations – one series is shown in table 1. Clearly calcium and magnesium ions enhance the rate of cleavage of the insulin molecule whereas cobalt, manganese and zinc are inhibitory. It has not been

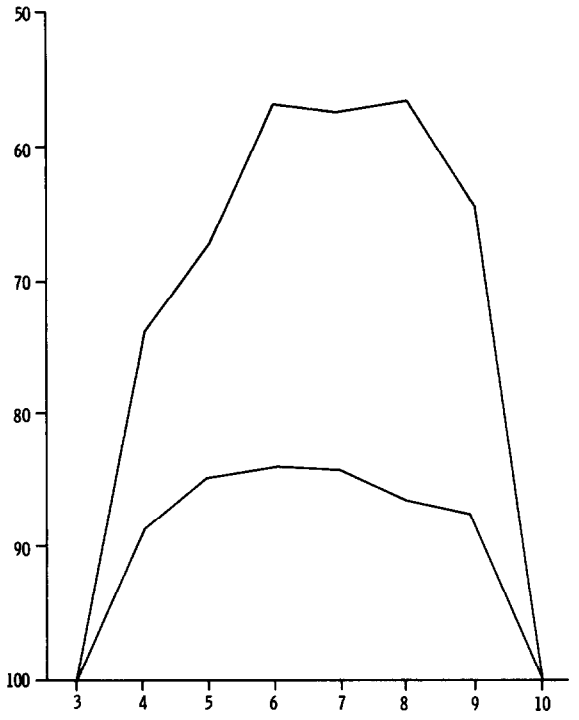

Fig.2. Influence of pH upon the cleavage of insulin by the protease from *A. Mellea*; lower line after 1 hr, upper line after 4 hr.

established whether these effects are due to interaction with the enzyme, with the insulin or with both.

Both porcine and bovine insulins have been treated with enzyme on several occasions on the several hundred milligram scale. Concentrations up to 100 mg/ml were used with either buffered solutions or with the pH held at the chosen value by the automatic addition of sodium hydroxide. The enzyme substrate ratio of 1:1000 was usually adhered to, sometimes calcium or magnesium ions were used, and temperatures were maintained between 35–40°C. The progression of the reaction was followed by automatic analysis of aliquots to determine both the amount of insulin and of the two products. Analysis took approx. 90 min and the reactions were terminated when all the insulin had been digested. The digests were purified by gel chromatography and, as with insulin [3], it was found that the 49 residue molecule behaved as the monomer in aqueous acetic acid. At the conclusion of the reaction the digest was immediately adjusted to a 20% solution in acetic acid and purified using Sephadex G-50 in 20% acetic acid solution. Comparable recoveries

Table 1
Effect of metal chlorides upon the AM protease–insulin reaction

	Molarity			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Ca^{++}	82	78	79	64
Mg^{++}	88	76	77	59
Co^{++}	13	14	42	69
Mn^{++}	10	12	47	45
Zn^{++}	0	0	36	34

Percentage reaction after 4 hr; control value 61%.

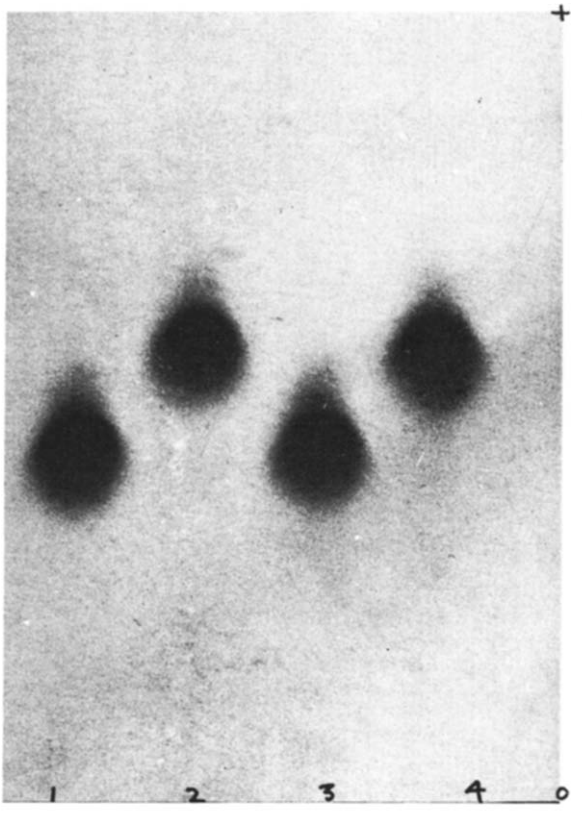

Fig.3. Acrylamide gel electrophoresis in Tris–HCl buffer pH 8.9 with staining by amido black of (1) bovine insulin (2) deslysylalanyl bovine insulin (3) porcine insulin (4) deslysylalanyl porcine insulin each previously purified by gel chromatography.

of over 90% were obtained using insulin and deslysyl-alanyl insulin after gel chromatography. Amino acid analyses of acid hydrolysates showed the complete absence of lysine in the products from both bovine and porcine insulins. Dansylation showed only N-terminal phenylalanine and glycine together with the *O*-tyrosine derivative. Thin-layer acrylamide gel electrophoresis at pH 8.9 showed that the modified peptides moved faster to the anode than the insulins themselves (fig.3). Trace spots which moved marginally faster than the main ones probably represented the desamido insulin in which aspargine 21 was replaced by aspartic acid; this feature would be retained after enzymic modification.

Impurities present in insulin preparations may well be involved in the production of antibodies to the insulins. All the lysine containing peptides examined so far are broken by the enzyme; glucagon, for example is rapidly degraded to give two smaller fragments. There is every reason to expect, therefore, that the lysine containing contaminants of the insulin preparations would also be degraded thereby allowing cleaner preparations to be obtained.

The two insulin derivatives showed similar effects upon blood sugar levels as the parent molecules using a rabbit hypoglycaemic assay (US Pharmacopeia Vol. 18, D. C. Earl, personal communication) and would therefore be acceptable alternatives on the grounds of potency.

Attempts have been made to show that the enzyme was completely separated from the products by gel chromatography and to see if the enzyme could be re-used. These are important points for any practical application of the process. Using a mild method of iodination [13], about 2 μg of enzyme was labelled with ^{125}I and purified using Sephadex G-25. Additional enzyme was added to the radioactive material and then this was subjected to the acetic acid Sephadex G-50 gel chromatography process. The recovered radioactive enzyme was used to digest porcine insulin under the usual conditions apart from a higher enzyme substrate ratio 200 μg to 50 mg insulin. The digest was resolved by gel chromatography (fig.4) and the enzyme fractions were combined and lyophilysed. The recovered enzyme was used to digest a second batch of insulin (20 mg), recovered as before and again used with porcine insulin (20 mg). In the latter case less than 10% of the insulin remained after

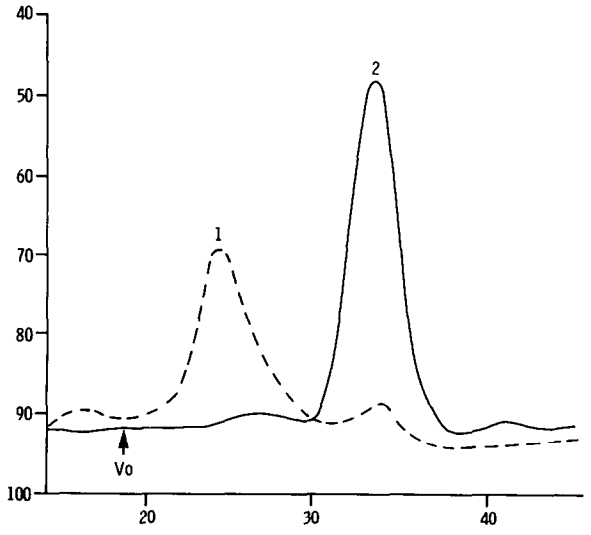

Fig.4. Separation of deslysylalanyl insulin (2) and ^{125}I labelled enzyme (1) using a column (160 × 2 cm) of Sephadex G-50 in 20% aqueous acetic acid at 4°C.

12 hr incubation. This does not of course provide an accurate measurement of the potency retained by the enzyme but does indicate that it is sufficiently robust to remain at least partially functional through several cycles of 20% acetic acid at 4°C, lyophilysations and incubations at pH 8 at 37°C. Gel chromatography separated enzyme and insulin efficiently but a small amount of radioactivity ($\sim$ 5%) remained in the insulin area after the first cycle but was not apparent in subsequent cycles. This could be reduced considerably by simple reprecipitation of the deslysylalanyl insulin. Attempts to obtain crystals from the 1–49 residue peptide have failed so far using conditions under which insulin itself crystallised readily [14].

These experiments show that a complete and specific cleavage of the C-terminal dipeptide of the B chain of insulins can be achieved using the protease from *Armillaria Mellea*. Gel chromatography provides a simple method of isolating the 49 residue products, and studies are proceeding on their capacity to cause a lower production of insulin binding antibodies.

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